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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) During the final period of the project, we were focusing on creating the model systems for the <i>in vitro</i> gene therapy experiments. The critical elements of this system are 1) the gene construct, 2) the gene delivery methodology, and 3) the <i>in vitro</i> skin model. We tested and compared the available delivery techniques by performing a series of transfection experiments using reporter gene systems (such as GFP) or antibiotic selection to assess efficiency. We worked extensively on development of the <i>in vitro</i> skin model, and succeeded in generating recombinant skin between keratinocytes and dermal cells in skin equivalents. To extend these studies and make them more widely applicable for wound care enhancement in blistering diseases as well as in chemically induced wounding, we went on to develop a model for <i>in vitro</i> epithelial reprogramming, in which we have begun to utilize different epithelial cell types as donor cells, in addition to keratinocytes. Collectively, we have shown that the use of gene delivery combined with epithelial cell-skin equivalent models, show significant promise toward developing a cellular 'bandage' for both genetically and chemically induced skin blistering.				
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4. Introduction

Several lines of evidence about the plasticity of stem cells (Lagasse et al, 2000; Lake et al, 2000; Morrison, 2001; Blau et al, 2001) has prompted our laboratory to consider **a novel approach to the treatment of inherited skin disorders such as EB, as well as in the treatment of chemically induced burns from sulfur mustard.** As in other fields, gene therapy in the skin has been hampered by the inability to target (or identify) a stem cell, and the lack of sustained gene expression. Rather than focusing on the identification and targeting of a stem cell in the skin, in the third year of this award, **we have recently initiated a new line of experimentation aimed at testing the hypothesis that epithelial cells from other sources might be coaxed into becoming skin cells given the right microenvironment.** Recently, for example, it was shown that adult rabbit cornea cells can be reprogrammed into skin under the right inductive dermal influences (Ferraris et al, 2000). Based on this evidence and other experiments performed in the first two years of this award, **we have begun to define the conditions under which reprogrammed cells might serve as a source of donor cells for therapy in genetic skin disorders and in the treatment of chemical burns.**

In years 1 and 2, we have developed an *in vitro* model using dermis as the inductive source, and have since demonstrated that we can induce skin-specific keratin gene expression from several primitive epithelia, such as amnion and cornea. In this final award period, **we will be focused upon the refinement of this model and**

determination of the pattern and chronology of gene expression changes corresponding to the reprogramming of amnion or cornea into epidermis.

We will also test whether we can recapitulate the *in vitro* results in an animal model. We will test this approach using an *in vivo* model first with immunodeficient mice, and finally with wild-type recipients to determine whether the grafts can be induced *in vivo*. Importantly, we will learn whether any rejection occurs between matched and unmatched donors and recipients, respectively.

This approach would offer significant advantages over the conventional gene replacement strategies that were proposed originally, which are currently dependent on the introduction of the exogenous gene as well as the ability to generate long-term expression and engraftment via stem cells. If these studies are successful, in the future we will ask whether the *in vivo* results can be replicated in normal human subjects. Historically, both amnion and cornea have been used safely in site-specific transplantation studies between unmatched individuals without rejection. Using donor cells from skin or other body sites of immunologically compatible healthy individuals would overcome both of these obstacles, since the **gene of interest would already be present**, and the exogenous tissue would be induced to form new skin tissue, and in so doing, **sequester a new population of stem cells.**

We believe this program of cellular transplantation and reprogramming holds great promise toward the eventual goal of successfully treating a broad

spectrum of genetic disorders of the skin, including EB, as well as the development of universal bandages for use in chemically-induced or other types of burns.

Background Studies

The major emphasis of several laboratories in epidermal biology, including our own, is on developing gene therapy approaches for skin diseases. As in other fields, gene therapy in the skin has been hampered by the inability to target (or identify) a stem cell, and the lack of sustained gene expression. We instead asked whether we could identify an ectopic source of epithelial cells that could be induced into becoming a skin stem cell. **Rather than searching for markers of the epidermal stem cell itself, we asked whether we could reprogram other epithelia into skin under the appropriate inductive (dermal) influences.** Two such candidate epithelial tissues are the cornea and amnion, since both have been used extensively in transplantation studies in the past, and since existing evidence suggests that both tissues demonstrate plasticity and the ability to be reprogrammed.

Much work is currently focused on using the skin as a donor tissue of stem cells for other diseases (neurological, muscular) (Toma et al, 2001), however, little interest is focused on **how to induce other cell types to become skin.** We reasoned that if the donor cells were taken from an immunologically-compatible individual or did not elicit an immune response, **such cells could overcome the two major obstacles in gene**

therapy approaches: gene introduction and targeting the stem cell. Donor cells, by definition, would contain an intact gene-of-interest, and importantly, others have shown that epidermal stem cells would be sequestered during the induction of the new skin and hair follicle, thus providing a lifelong supply of genetically corrected cells.

Use of Amnion in Transplantation Biology

Amnion has been widely reported as a biological dressing for burns and its advantages have been well-documented in the literature (Maral et al, 1999, Ruzczak and Schwartz, 2000). It eliminates pain, allows wounds to dry faster and promotes early epithelialization. **Amnion is inexpensive to use, easily obtained and stored, it has antimicrobial properties and low antigenicity, all of which contribute to its utility as a wound dressing,** particularly in developing countries. Several different preparations of amnion have been used, including fresh, frozen, dried, irradiated, lyophilized and glycerolized. In one recent study, preserved amnion was applied to split-thickness skin graft donor sites in five patients (Maral et al, 1999). Wounds were covered with nonadherent gauze and left undisturbed, and the wounds were completely epithelialized after 10 days. **Importantly, no evidence of acute or late rejection was observed in any of the subjects (Maral et al, 1999).**

In addition to its application as a wound covering, amnion as well human amniotic epithelial cells (HAE) themselves have attracted interest as potential donor cells for the treatment of metabolic disease (Akle et al, 1981, 1985; Scaggiante et al, 1987). In

particular, they were tested as donor cells for treatment of lysosomal storage disorders including Niemann-Pick Disease and mucopolysaccharoidoses such as Hunter's and Hurler's Diseases. In both studies, repeated implantation of amnion or HAE cells was used as a source of enzyme replacement, and no evidence of immune response toward the transplanted cells was observed.

Historically, the question of the immunogenicity of transplanted amnion has been the subject of much study. It has been shown that HLA-A, B, C and DR antigens are not expressed on freshly collected or cultured HAE cells (Adinolfi et al, 1982; Yeh et al, 1983). Transplantation of amnion into allogeneic hosts does not result in overt acute graft rejection. In one study, HAE were implanted subcutaneously into the arm of seven volunteers, and again, no evidence of acute reaction was present, along with no pain or redness of the skin (Akle et al, 1981). If there was any immune response to the implants, it was low grade and chronic rather than active. In addition, it did not result in rejection of the HAE cells, since they appeared to survive, and in some cases, even proliferate beneath the skin.

Amnion has also been used as a biomaterial in a number of different surgical applications. In microvascular surgery, human amnion has been studied as an acceptable substitute to autologous vein (Gray et al, 1987). Amnion-derived interpositional grafts were shown to have a patency rate similar to that of autologous vein grafts, healed and re-endothelialized within 3-4 weeks, and

importantly, were not rejected by the recipient rats. Amnion has also been used in the surgical treatment of congenital absence of the vagina in a series of 21 patients (Tozum, 1976). The transplanted amnion was found to form a mitotically active, proliferating squamous vaginal epithelium. The authors report that "(Amnion) has a superb capability for regeneration and metaplasia. Since it is embryonic tissue which activates proliferation and regeneration of the cells adjacent to it, it is accepted and not rejected by the recipient. Eventually these cells respond to exogenous estrogens by mitosis and maturation of an epithelium" (Tozum, 1976). Finally, HAE cells have been used as donor cells for reestablishment of a damaged ocular surface (He et al, 1999). Following transplantation of HAE cells onto denuded corneas, they were found to repolarize and tightly adhere to the underlying stroma via newly formed hemidesmosomes, suggesting they are capable of generating specialized keratinocyte-like attachment junctions.

More recently, HAE cells have received renewed attention in cellular therapy because of emerging evidence about the general plasticity of stem cells (Morrison, 2001; Blau et al, 2001). The amnion is the inner layer of the fetal membranes and is contiguous with the ectoderm of the embryo. It is composed of a single layer of cuboidal or flattened epithelial cells on the inner surface and a mesenchymal connective tissue layer on the outside. At about 8 days after fertilization in humans, a small cavity appears within the epiblast that enlarges to become the amniotic cavity. HAE cells are formed from amnioblasts adjacent to the cytotrophoblasts, and line the amniotic cavity

as well as the rest of the epiblast. For this reason, **the hypothesis has been put forward that HAE cells may have the potentiality to differentiate into various organs including the heart, brain and liver, given the correct microenvironment** (Sakuragawa et al, 1996). Several studies have since shown that HAE cells may have the putative multipotentiality of neurons, astrocytes and oligodendrocytes, and express markers for both neuronal and glial cells (Sakuragawa et al, 1996, 1997). Further, HAE cells have shown evidence for acetylcholine metabolism, suggesting that they could be applied for intracerebral allografting in neurologic disease in which cholinergic neurons are damaged (Sakuragawa et al, 1997). The same authors have since successfully used HAE as donor cells in a model of brain ischemia, demonstrating that HAE may have therapeutic potential for the treatment of ischemic damage in neuronal disorders (Okawa et al, 2001). **Collectively, these studies have begun to address the question whether amnion can adopt different cell fates in an ectopic environment.**

Differentiation of Rat Amnion into Epidermis

The differentiation potential of rat amnion was explored in a study in which the authors sought to determine the lability of amnion by placing it into three ectopic body sites: 1) the kidney capsule; 2) subcutaneous on the back; 3) wrapped in omentum, and also in an in vitro culture model (Knezevic, 1996). In this study, the amnion was not placed on an apposing source of mesenchyme, but instead was transferred or cultured by itself, thus, there was no specific 'message' instructing the amnion to adopt a particular cell fate. **Interestingly, it was discovered that in each of the three body**

sites as well as the culture model, the amnion had spontaneously differentiated into skin, and in some cases had formed skin appendages such as hair follicles and sebaceous glands. The formation of hair follicles also suggests that a **new population of stem cells** had been sequestered simultaneously with appendage formation. The authors suggested that the **differentiation of both amniotic ectoderm and embryonic surface ectoderm into skin appear to be morphologically related phenomena**, perhaps via a common developmental pathway (Knezevic 1996).

Cornea Transplantation

Cornea transplantation is the oldest, most common, by far the most successful form of tissue transplantation (Niederkom, 1999). In the United States alone, over 40,000 corneal transplantations are performed each year. Remarkably, however, less than 10% of uncomplicated, first-time cornea transplants will undergo immune rejection even though HLA matching is not routinely performed and the use of immunosuppressive drugs is limited to only topical corticosteroids. The success of corneal transplantation predates the use of corticosteroids, and thus further underscores the remarkable privilege of corneal allografts. The explanation for the immune privilege of corneal allografts is based on the obvious avascularity of the cornea, which is believed to somehow sequester the graft from the induction of allodestructive immune responses (Niederkom, 1999). The success of this therapy is dependent on the gradual replacement of the donor's corneal epithelium by the

recipient's healthy limbal (stem) cells, and the persistence of donor cells has not been widely studied.

Another group of disorders, the ocular surface diseases, is characterized by the depletion of the stem cell population from the corneal limbus. Thus, conventional cornea transplantation is not successful in these patients since the donor cornea is unable to be repopulated by the recipient's limbal cells. It has recently been shown that sufficient stem cells can be derived from a small 2 mm limbal biopsy and expanded *ex vivo* by 100 fold ($1-2 \times 10^7$ cells) to create a graft that is easily transplantable and biologically mimics the corneal surface (Schwab et al, 2001). The small biopsy from the (autologous or allogeneic) donor eye is not sufficient to cause long term damage and does not put the eye at risk. The authors noted that as the limbal cells were cultured, 2-9% remained as stem cells through the cultivation process (Schwab et al, 2001). The survival of expanded limbal grafts suggests that both the limbus and the central cornea were regenerated, indicating that limbal cells were reseeded as stem cells when placed back into the potentiating microenvironment.

Reprogramming of Rabbit Cornea into Epidermis

Recently, the first evidence for reprogramming of corneal epithelium into skin was reported using recombinant models of rabbit cornea and mouse embryonic dermis (Ferraris et al, 2000). The authors of this study sought to demonstrate the plasticity of corneal epithelium in response to a new mesenchymal signal. Importantly, the authors

used central cornea (transit amplifying, or committed cells) rather than limbal (stem) cells to prove that central cornea could be reprogrammed by the dermis and switch from a cornea-specific keratin gene expression profile (K3/12) to a skin-specific keratin pattern (K5/14, K1/12). Furthermore, the reprogrammed cornea even produced hair follicles, pilosebaceous units and sweat glands. These remarkable results showed for the first time that a even differentiated epithelium could be reprogrammed, and suggested that **a new population of stem cells were sequestered in the newly formed appendages.**

Clinical and Military Significance

Based on these lines of evidence, in this year 3 of this proposal, we will test the hypothesis that **human cornea and amnion can be reprogrammed to become skin** and serve as a **biomaterial for gene therapy of human skin diseases and chemical injuries.**

Why would such a material be superior to conventional skin grafting for the treatment of genetic skin diseases?

First, autologous skin grafting on a patient with a genetic disease does not represent an improvement, since the donor (self) cells are also genetically deficient. Therefore, allografts could be used, however, historically these have been shown to be efficiently rejected by recipients. Artificial skin equivalents could be used, however,

these provide only a costly and temporary wound cover, and in normal individuals, their own keratinocytes simply use these dressings as a scaffold for re-epithelialization. However, in genetically deficient patients, such as junctional EB patients with BPAG2 mutations, wound healing and cell migration are among the major cellular defects, resulting in eventual loss of the skin equivalent.

In the acute treatment of burns in the military setting, there is little time to expand keratinocyte cultures and perform surgical skin grafting, though in extreme cases, this method is still utilized. **A modality which provided a 'living' bandage that was not rejected would represent a significant advance.**

Most importantly, none of these modalities offers **a means for repopulating the stem cell compartment of the skin**, and by definition then, eventually all donor cells, whether genetically correct or not, would be lost.

The major advantages of reprogrammed skin are the following:

1) The supply of **donor amnion** is limitless and inexpensive. Given the lack of immune response to the donor amnion in any of the studies mentioned above, it is likely that amnion or culture HAE cells will represent ideal donor tissues for reprogramming.

2) The supply of **donor cornea** and the techniques for limbal stem cell culture and manipulation are well-described, and the tissue is readily accessible. To avoid

immune rejection, donor limbal cells could be harvested from first-degree relatives of JEB patients who are only carriers of BPAG2 mutations, expanded and reprogrammed into skin.

3) A major advantage of using **autologous donor cells** is that, by definition, they are **genetically correct**, insofar as they do not have the same mutation(s) as the recipient. **Thus, one of the two major hurdles of conventional gene therapy approaches is overcome by this technique.** The wild-type BPAG2 gene exists in the donor genome in the proper transcriptional context and should not be subject to inactivation of expression.

4) Finally, the second major obstacle in gene therapy for skin diseases is the lack of ability to identify a stem cell. **The use of reprogrammed skin overcomes this hurdle as well**, since by definition, the induction of new hair follicles, pilosebaceous units and sweat glands would result in the partitioning of **a new compartment of epidermal stem cells** in the newly formed appendages.

In the final year of this proposal, we will further develop and perfect the techniques of both *in vitro* and *in vivo* reprogramming of amnion and cornea into skin. **Once we have established that there is no immune response in animal models, our future goal is to test the use of reprogrammed skin in a limited number of human subjects.**

If we are successful, this work will be expanded into a study aimed at establishing the utility of reprogrammed skin in the treatment of human genetic skin diseases, such as the different forms of EB, ichthyoses and epidermolytic hyperkeratosis, among others, as well as chemical injuries and burns. These disorders are currently the topic of much gene therapy research, however, largely by the conventional (and not yet successful) approaches of *ex vivo* cell culture, gene replacement, and re-grafting. Further, if these studies prove fruitful, we would also seek funding and approval to begin to test this approach in patients with chemical or other types of burns.

The success of this project would provide a foundation for the clinical application of reprogrammed skin in the treatment of acquired, environmental and genetic skin diseases.

5. Body

Progress in Task 3: In Vitro Model for Reprogramming of Epithelium

As an extension of our work during years 1 and 2, we have developed a newly described modified *in vitro* skin equivalent model (Szabowski et al, 2000; Maas-Szabowski et al, 2001) using embryonic mouse dermis as the inductive source. **Using this model, we have demonstrated that we can induce skin-specific keratin gene expression from amnion after two weeks in culture (Figure 1).** The

intact amnion is negative for keratin 1/10 staining, whereas after two weeks in culture, K1/10 staining is induced in the suprabasal layer of the dividing amnion (Figure 1).

a. Rationale and Experimental Design

We are focused upon the refinement of the model developed in years 1 and 2. In particular, we will use the model to determine of the pattern and chronology of gene expression changes corresponding to the reprogramming of amnion or cornea into epidermis. We will ask whether the disappearance of the characteristic amniotic keratin (K8) pattern or corneal specific keratin pattern (K3/12) occurs before, after, or at the same time as the appearance of skin specific keratins (K5/14 and K1/10). Importantly, we will lift the grafts to the air-liquid interface to determine whether we can induce terminal differentiation, and look for the appearance of markers such as loricrin and involucrin. We will also study the appearance of K19 as an indication of newly sequestered stem cells. We will initiate these studies using mouse embryonic fibroblasts as the source of inductive mesenchyme, and explore the use of other dermal cells, such as hair follicle dermal papilla cells, should the need arise.

For preparation of mouse embryonic fibroblasts, a pregnant female mouse (13 days pc) will be sacrificed with CO₂. 4-6 embryos are removed and washed in PBS. Liver, intestine, head and limbs are removed. After washing in PBS embryos are minced with scissors in trypsin/EDTA until it can be taken up in a 10 ml pipette. The mixture is pipetted up and down several times and incubated at 37°C for 10 minutes in a Petri dish. Pipetting with 5 ml pipette is followed by another 10 minutes of incubation.

Then, all contents are transferred into a 50 ml tube. The cellular debris is allowed to settle out over a period of 2 minutes. The supernatant is removed into a fresh tube, mixed with MEF media (up to 50 ml) and centrifuged at 1000 RPM for 5 min at 10°C. The pellet is re-suspended in MEF medium and cells are plated in 250 ml flasks. The next day the medium is changed to remove cellular debris and cells are incubated for another 24 h.

Rat tail collagen (Sigma #C7661) is dissolved in 12 mM HCL (4 mg/ml) and incubated at 4°C overnight for swelling. Then, one part of 10x Hank's balanced salt solution (Gibco #14180-061) is added to 8 parts collagen solution and pH set to 7.0 (on ice). The collagen is immediately mixed with one part MEF (mouse embryonic fibroblasts; 500,000 cells in 2 ml collagen) in 'skin model medium' (SMM). 2 ml of the final mixture is immediately poured in cell culture inserts placed previously in 6-well culture plates (Falcon, Multiwell tissue culture plate, # 3846) filled with 2 ml of SMM. After 1 h polymerization (at 37°C), SMM (2 ml) is added on the top of the gel.

Discarded human amnion tissue is obtained from recent (not more than 1 hour) Cesarean section, from the Department of OB/GYN at Columbia Presbyterian Medical Center. The mesodermal component was removed with forceps using a stereomicroscope. The ectodermal component of amnion was washed in PBS with 5x antibiotics and cut into pieces of approximately 2x2 cm. Pieces of amnion were placed on top of polymerized collagen (epithelial side up) and weighed down using sterile teflon rings. The system was incubated undisturbed for two days. After that, the

medium was removed from inside of the ring while it was kept outside the ring. Incubation was continued for 14 days. Central corneas and limbus cells will be harvested from adult mice, and either cultured or placed directly in the same in vitro model described. Immunohistochemistry will be performed every 2-3 days for 14-21 days using antibodies for K3/12, K8, K1/10, K5/14, loricrin, involucrin and K19 either from commercial sources (Sigma, Boehringer, Neo Markers) or through collaboration.

b. Outcomes, Expected Results and Alternatives

While it is anticipated that lifting the in vitro recombinant grafts to the air interface will induce differentiation, it is possible that reprogrammed amnion or cornea may not respond in the same manner as keratinocytes. If we encounter this dilemma, we will introduce compounds into the culture medium which have been shown by others to induce differentiation and barrier formation in fetal rats (Hanley et al, 1999; Billoni et al, 2000). These compounds include activators of the nuclear hormone receptor PPAR, in particular, clofibrate (1 mg) and linoleic acid (1 mg). Both agents have been shown to promote epidermal maturation, barrier formation and stratum corneum development, suggesting that they may be of use in promoting differentiation should this prove to be a challenge in our model.

We will begin these experiments using embryonic mouse dermis as the inductive source, since these cells were shown to be sufficient to reprogram

rabbit cornea in the kidney capsule model (Ferraris et al, 2000). Although unexpected, should these cells prove insufficient for induction of reprogramming in our model, we will substitute cultured human and/or rodent dermal papilla and/or dermal sheath cells as the inductive mesenchyme. In general, we expect to move from human, rat and mouse donor sources throughout the course of these experiments these experiments, at all times using discarded or leftover human amnion or cultured corneal cells. Dr. Colin Jahoda, a collaborator of ours on this project, is a long-standing expert in the field of epithelial recombinations and induction and will provide us advice on the donor tissues as well as specialized dermal cells as needed.

Continuation of Task 3: *In vivo* model of epithelial reprogramming

We will then test whether we can recapitulate the *in vitro* results in an animal model. We will use an *in vivo* model first with immunodeficient mice, and finally with wild-type recipients to determine whether the grafts can be induced *in vivo*. Importantly, we will learn whether any rejection occurs between unmatched donors and recipients. Our experimental plan involves the following strategies: 1) human amnion grafted onto SCID (Charles River C.B.-17/scid) mice; 2) human amnion grafted onto wild-type C57BL/6 mice; 3) cultured C57BL/6 mouse cornea/limbus grafted onto SCID mice; 4) cultured C57BL/6 mouse cornea/limbus grafted onto wild-type C57BL/6 mice; 5) cultured BALB/c cornea/limbus grafted onto C57/BL6 mice. Grafts will be analyzed

for microscopic and macroscopic criteria, and the changes in gene expression defined earlier, as well as for any signs of immune response or rejection.

a. Rationale and Experimental Design

We will introduce 2x2 cm full-thickness wounds in 15 mice in each of the five groups by surgically excising the skin. Under aseptic conditions, the skin will be excised to the level of the muscle with careful hemostasis. Wound margins will be tattooed with India ink, allowing for photometric and visual standardization and sequential photographs. The wound area will be covered with fresh amnion or cultured cornea cells or left open as a control wound. The graft will be sutured to the wound margin and covered with a nonadherent semi-occlusive gauze dressing. When needed animals will have wound chambers to protect the wounds. Wounds will be evaluated macroscopically and microscopically after 2, 4, 5, 10 and 14 days, three different mice per examination for each type of wound. The primary adherence or 'take' of the graft will be assessed at 4 and 10 days by gross inspection and histology.

All grafts will be scored based on three macroscopic (adherence, color, pliability) and three microscopic (structural integrity, leukocyte infiltration, adherence) criteria. The Kruskal-Wallis test will be used to statistically compare the total performance scores of the different groups. The Mann-Whitney test will be used to compare one group to another when the difference among groups is found to be significant (Dawson-Saunders and Trapp, 1990). In addition to these tests, immunohistochemistry will also

be performed with all markers specified earlier, as well as careful assessment for immunological markers if necessary. Here we will look for evidence of stratification, expression of skin specific keratins, terminal differentiation markers, as well as immunological evidence of rejection versus tolerance.

We predict that skin appendages such as pilosebaceous units will be present in the reprogrammed skin on the basis of previous studies (Ferraris et al, 2000). To help us evaluate this possibility, we have again enlisted the expert help of Dr. Colin Jahoda, University of Durham, UK, who conducted the rabbit cornea experiments referenced above.

b. Outcomes, Expected Results and Alternatives

We anticipate that there may be some infiltration of the grafts by surrounding keratinocytes during normal wound healing. Therefore, to prevent this from occurring and to allow the transplanted cornea or amnion the maximal opportunity to adhere and 'take', we will introduce the use of a wound chamber embedded subcutaneously to prevent inward migration of keratinocytes. We will use either a modified chamber (P.A. Medical, Columbia, TN) which consists of a flexible vinyl or silicon ring bonded to an adhesive base. We will attach the adhesive ring to a flat base which will be implanted subcutaneously and form a physical barrier, preventing the migration of keratinocytes into the graft. A similar

modified system was recently reported by Mizoguchi et al. (Mizoguchi et al, 2001) for use in implanting human skin equivalents onto the back of nude mice.

While the initial wound coverage will be nonadherent gauze dressing, clearly there can be significant differences in wound healing depending upon the choice of dressing. Should the need arise and we encounter difficulties, we will also attempt different dressings including polyurethane foam (Allevyn), paraffin gauze, polythene sheet (Opsite) and silicone sheets if necessary. In addition, the timing of exposing the wounds to the air versus allowing for wound dessication will also be taken into account when developing the protocol for wound coverage which best promotes epithelial reprogramming.

We have proposed the initial experiments to be performed in mice. Given the difficulties in culturing mouse keratinocytes, we are aware that there may be technical challenges involved in establishing culture conditions from corneal cells as well. Should we encounter difficulty in the culture or propagation of mouse corneal cells, or the wounds be of insufficient size, we will convert the experiments to be carried out in the brown Norway as wild type and Charles River immunodeficient rats (CrI:Rnu BR (Nude) as recipients. We will move freely between donor human, mouse and rat tissues in both wild-type and immunodeficient recipients, to best assess the questions of rejection versus tolerance.

6. Key research accomplishments

In this final year of funding, we have greatly expanded the scope of the initial skin equivalent experiments proposed, to include the use of alternative epithelia such as cornea and amnion as the source of reprogrammed keratinocyte precursors.

We have been working extensively on the *in vitro* skin model described in the proposal initially. We first proceeded with the assembly of a model consisting of normal cells, to be used as a control during the recombination experiments. According to the experimental outline, we performed a two-step assembly in all cases. During the first phase, we developed a multi-layer fibroblast base, which served as a recipient surface for the keratinocytes, amnion or cornea in the second phase. After successful attachment of the seeded epithelial cells, the system was elevated to the air-liquid interface to allow the multi-layer growth and differentiation of the epithelium.

We built the skin models using different combinations of starting materials. All culture systems were initially based on a collagen matrix, which contained a variable proportion of fibroblasts. Their attachment, cell division and growth characteristics were observed and evaluated. The human cell based models were compared side-by-side with skin models utilizing cell types of murine origin. In the *in vivo* experiments, we will use inductive dermis as the reprogramming source and these experiments will be expanded as outlined in the continuing experimental plan.

7. Reportable Outcomes

Preliminary data from these studies served as the basis for an **NIH R21 grant that was recently funded** focused on epithelial reprogramming as an alternative gene therapy approach.

8. Conclusions

Upon successful completion of this project, we hope to have acquired sufficient preliminary data with which to justify a trial of reprogrammed skin in patients with genetic skin disorders in the future. While this study has evolved rapidly, keeping pace with the stem cell field around it, we have never lost sight of our **original stated goals of developing a cellular therapy for EB and for chemical burns**. At the outset, we could have never predicted the speed with which the gene therapy and tissue engineering fields have advanced. We have done our very best to be sure that the goals of the US Army and Medical Research and Materiel Command were not only met, but we believe, exceeded.

Finally, we are extremely proud to report that this recent work focused on epithelial reprogramming as an alternative gene therapy approach **was recently**

funded (on its first review) by the NIH as an R21 award to continue to advance this very exciting and promising preliminary data.

We are enourmously grateful for the generous support of the USAMRC during this initial three year period. Particularly in light of the events of September 11, 2001, we have been deeply honored to serve in this important capacity. Due to your support, our progress has been swift toward the goal of enhancing the military readiness of chemical injuries. Should the USAMRC deem that our work is worthy of continuation by a future funding mechanism, we would again be very proud to continue with this work.

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10. Appendices

Figure 1.

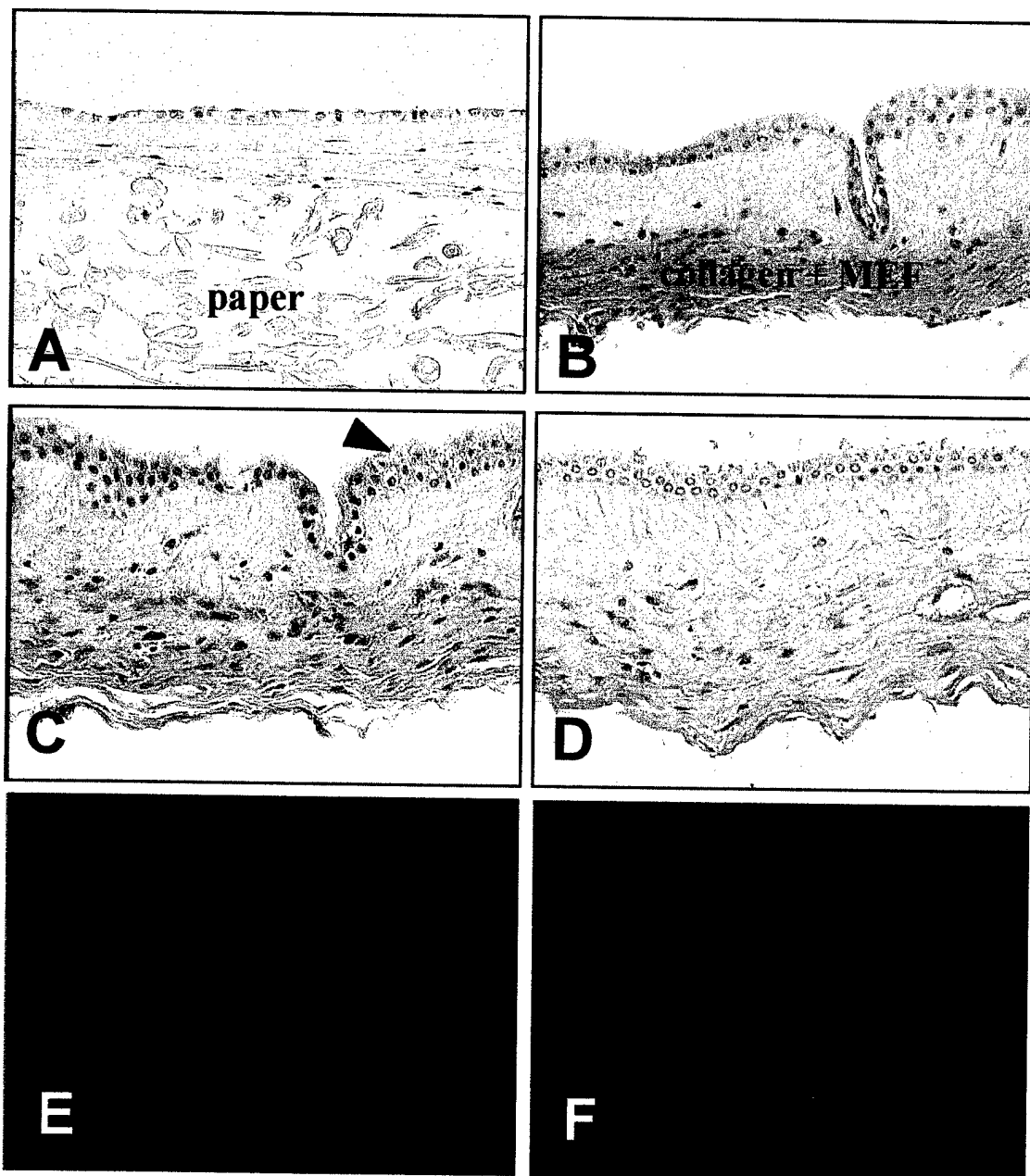


Figure 1. (A) Intact amnion on filter paper. (B) Amnion after one week of culturing on collagen bed (+ mouse embryonic fibroblasts). Fibroblasts penetrate the amniotic membrane. Amnion and collagen/fibroblast system is completely fused. Amnion cells proliferate and form 2-3 layered structure. (C) Amnion after one week in culture. Membranes of some cells in the upper layer are damaged (arrowhead). (D) Two weeks of culturing. (E) Intact amnion on paper. Keratin 1/10 immunohistochemistry. No positive staining is present (some non-specific fluorescence of the paper bundles is visible in the lower part of the picture). (F) Amnion after two weeks of culture. Cells of the upper layer become K1/10 positive.